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DUSTIN J. PENN AND KERSTIN MUSOLF

Introduction

The existence within natural populations of large amounts of genetic variation in molecules and morphology presents an evolutionary problem. (Clarke, 1979)

The genes of the major histocompatibility complex (MHC) appear to be the most polymorphic loci in vertebrates, and explaining how natural selection maintains such genetic diversity ('population allelic richness') is a major unsolved problem in evolutionary biology. There is accumulating evidence that MHC polymorphisms are maintained by balancing selection (Apanius *et al.*, 1997; Spurgin and Richardson, 2010); however, the nature of this selection is still unclear. MHC genes encode cell-surface glycoproteins (class I and II molecules) that present peptide antigens to T cells, and thereby play an important role in the development of the T cell receptor (TCR) repertoire, immunological self/non-self recognition, and resistance to pathogens and parasites. Many MHC alleles increase susceptibility to infectious and autoimmune diseases, which makes MHC polymorphisms especially puzzling, as these harmful alleles should be eliminated by natural selection. The evolutionary *origin* of MHC diversity is generated by mutation, recombination, and gene conversion (Martinsohn *et al.*, 1999); however, these mechanisms do not explain how selection maintains polymorphisms. In this chapter, we review theoretical and empirical studies on the evolution of MHC diversity, with a particular focus on house mice (*Mus musculus*).

There are two general hypotheses proposed to explain how selection maintains MHC polymorphisms. One hypothesis suggests that MHC polymorphisms are maintained by selection from pathogens and parasites (pathogen-mediated selection (PMS)) (Apanius *et al.*, 1997; Spurgin and Richardson, 2010). This idea is the most likely explanation, given the role MHC molecules play in immune recognition of pathogens. The most viable models for PMS include negative frequency-dependent

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selection (FDS) and fluctuating selection (FS) (Hill *et al.*, 1991; Hedrick, 2002), though direct evidence for these hypotheses is lacking. If pathogens are the *only* factor driving MHC diversity, however, then most other immunological receptor loci should also be highly polymorphic (Penn and Potts, 1999). As MHC genes appear to have exceptional polymorphism, it is crucial to consider other sources of selection.

A second general hypothesis suggests that MHC diversity is maintained through sexual selection (non-random mating) (SS). Studies in house mice, and an increasing number of other species, indicate that MHC genes influence odour and mating preferences (Penn and Potts, 1999; Penn, 2002; Yamazaki and Beauchamp, 2007; Radwan *et al.*, 2008; Schwensow *et al.*, 2008; for humans, see Havlicek and Roberts, 2009). MHC-disassortative mating preferences are sufficient to drive MHC polymorphisms (Hedrick, 1992), and there are several possible reasons why such preferences evolved. MHC-dependent mating preferences may function to enhance offspring resistance to pathogens, avoid inbreeding, or both (Penn and Potts, 1999; Penn *et al.*, 2002; Piertney and Oliver, 2006). In addition to increasing offspring MHC and overall heterozygosity, MHC-disassortative preferences may function to optimize offspring MHC heterozygosity or provide a 'moving target' to protect offspring against rapidly evolving pathogens (Penn and Potts, 1999). The PMS and SS hypotheses are sometimes mistakenly assumed to be mutually exclusive alternatives; however, these ideas are completely compatible. In fact, there are several reasons to expect PMS to selectively favour the evolution of MHC-dependent mating preferences (Penn and Potts, 1999).

In this chapter we provide an integrative overview of the evolution of MHC diversity (for other reviews, see Apanius *et al.*, 1997; Edwards and Hedrick, 1998; Penn and Potts, 1999; Meyer and Thomson, 2001; Penn, 2002; Bernatchez and Landry, 2003; Garrigan and Hedrick, 2003; Sommer, 2005; Milinski, 2006; Piertney and Oliver, 2006; Spurgin and Richardson, 2010). Recent reviews on this topic focus on 'non-model' species, whereas here we focus mainly on house mice (*M. musculus*), an important model species for MHC research (Box 9.1). We summarize surveys of MHC polymorphisms, and we consider a related problem, the evolution of duplications. We consider both PMS and SS hypotheses, and address misunderstandings about the SS hypothesis. Finally, we provide ideas for the future and explain why more studies are needed on selection on MHC genes in more natural ecological conditions.

MHC polymorphisms

> This degree of polymorphism is unprecedented; there is no other locus whose polymorphism comes anywhere near that of the HLA-A,B and H-2K,D loci. (Klein, 1987)

Box 9.1 House mice as a model organism in MHC research

Domesticated house mice are the premier model species for biomedical research, and have long played an important role in MHC research (Klein, 1975, 1986; Melvold, 2001; Penn and Ilmonen, 2005). MHC genes were first discovered in research on tissue rejection using domesticated strains of house mice. In the 1930s, Peter Gorer found mouse blood group antigens, which he called 'antigens I–IV' and George Snell and his colleagues later bred the first congenic strains of mice and showed that tissue rejection is controlled by multiple closely linked loci, rather than a single locus (MHC genes in mice are often called '*H2*' genes, a term that originated by combining Snell's 'histocompatibility (H) genes' with Gorer's 'antigen II', whereas in humans they are often called 'human leucocyte antigen' (HLA)). Subsequent studies with house mice have led to some of the most important discoveries in immunology, including the finding that MHC genes control immune responses to antigens (McDevitt and Benacerraf, 1969; McDevitt and Chinitz, 1969) and the development of the TCR (Zinkernagel and Doherty, 1975, 1997). The unusually high levels of polymorphisms were first described in wild populations of house mice (Klein, 1970; Klein and Bailey, 1971). In more recent years, the development of mutant, transgenic, and other strains of mice have enabled researchers to examine how MHC genes affect disease resistance, including immune resistance to infection, tumour surveillance, autoimmunity, and other forms of immunopathology. Genetic techniques that allow disruption or insertion of particular MHC and other loci (so-called genetic 'knock-outs' and 'knock-ins') provide state-of-the-art methods (Shastry, 1995; Wolfer *et al.*, 2002). An amazing number of MHC congenic, transgenic, and other mouse strains are available from JAX labs and other commercial suppliers, and these mice have become central tools for biomedical research (Crawley, 1999, 2000). Unfortunately, however, there has been comparatively little work conducted on MHC – or other genes – in populations of wild house mice to understand how natural selection maintains the diversity of these genes.

There are five closely linked classical, antigen-presenting, class I and II MHC loci in house mice (Box 9.2), and population surveys find extensive genetic polymorphisms at these loci, with over 100 major alleles per locus (Klein, 1970; Duncan *et al.*, 1979a; Klein and Figueroa, 1981). A typical house mouse population will carry more than a dozen alleles per locus, as with humans and many other species. However, levels of variation vary between loci of different classes and among the different chains (α and β) of class II – e.g. Eα shows reduced polymorphisms (Duncan *et al.*, 1979b) – and MHC alleles or their frequencies differ among neighbouring populations in spite of a social structure that would tend to reduce heterozygosity (Duncan *et al.*, 1979a). In addition to being polymorphic, MHC genes also show unusually even distributions of allelic frequencies (indicating balancing selection) (Nadeau *et al.*, 1988) and large sequence divergences among alleles (suggesting ancient origins) (Apanius *et al.*, 1997; Hughes and Yeager, 1998; Klein *et al.*, 2007).

It has been suggested that MHC polymorphisms can be used for estimating the size of the founding population of new species and analysing the long-term population demographics of phylogenetic lineages (Klein *et al.*, 2007). However, to distinguish demographic from selective processes, one must use many unlinked loci, because a bottleneck will affect the entire genome, whereas a selective sweep affects some loci more than others (Galtier *et al.*, 2000).

Box 9.2 Genetic architecture/genomic organization (H2, haplotypes, and strain nomenclature)

The MHC as an organizational unit originated during the evolution of jawed vertebrates (Kelley *et al.*, 2005). Its genomic organization appears conserved in many species, as linkage between the class I and class II regions is present from cartilaginous fishes to humans, with the exception of bony fishes (Kulski *et al.*, 2002). In mice, MHC (also known as '*H2*') genes are located on chromosome 17. On the basis of distinct structural and functional characteristics, MHC genes are divided between class I and II molecules (so-called class III genes are not shown in Fig. 9.1). Adjacent to the classical class I and II MHC molecules, other homologous genes are non-classical MHC genes, though their functions are not as well understood. The classical H2 complex (see Box 9.1) in mice is small, with three class I loci (K, D, and L) and two types of class II loci (A and E), each further divided into their respective α and β chains. Recombination within is relatively rare, and therefore a set of alleles is usually transmitted together as a unit called a haplotype. This is indicated in laboratory strain nomenclature by superscript designations (e.g. $H2^b$, $H2^d$, $H2^k$). Within one haplotype, individual alleles have been correspondingly designated with respect to their original locus (e.g. K^d, $A\beta^d$, $A\alpha^d$, $E\beta^d$, $E\alpha^d$, D^d, and L^d). Through recombination events, haplotypes occur with alleles derived from different original sources (see Table 1 in Stuart, 2010). Interestingly, a recent study comparing two closely linked MHC class II genes across the European house mouse hybrid zone demonstrated that despite their tight linkage, both loci showed disparate evolutionary patterns (Čížková *et al.*, 2011), indicating the need for multi-locus analyses.

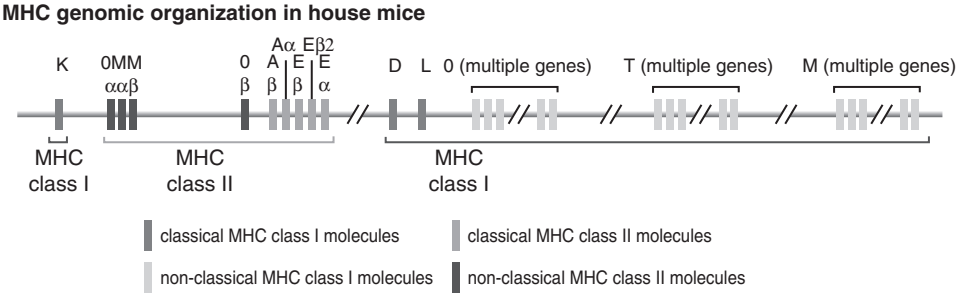

Figure 9.1 MHC genomic organization in house mice (modified with permission from DeFranco *et al.*, 2007). See plate section for a colour version of this figure.

Klein and his colleagues (1987, 2007) surveyed MHC diversity in wild house mice and discovered surprisingly poor consistency between the phylogeny of MHC alleles and phylogenetic trees that reveal the evolutionary history of different *Mus* species. One expects that MHC alleles would be more closely related to alleles within than among species, and yet they often show just the opposite pattern. To explain this odd finding, Klein and his colleagues suggested that MHC allelic lineages are ancient and predate speciation (cladogenesis) events. Such 'trans-species polymorphism' is defined as the passage of allelic lineages from ancestral to descendant species (Klein, 1987; Figueroa *et al.*, 1988; Klein *et al.*, 2007). Subsequent findings in rodents are mixed, with evidence for ancestral polymorphism of MHC class II genes in the genus *Mus* (Edwards *et al.*, 1997) and *Apodemus* (Musolf *et al.*, 2004), but not in *Peromyscus* (Richman *et al.*, 2003), suggesting that variable selection pressures have contributed to the evolution of MHC in rodents. For cases of trans-species polymorphisms it appears as if selection has maintained MHC allelic lineages for tens of millions of years, which seems improbable. Another possibility is that these patterns may be due to gene conversion (segmental exchanges of sequence motives between alleles of the same or different loci; Martinsohn *et al.*, 1999). Results from comparative phylogenetic analyses have suggested that interspecific allele sharing is generated by a recent origin and independent convergent evolution from similar selection pressures from pathogens, rather than common ancestry (Yeager and Hughes, 1999). If so, this would require widespread independent convergence in DNA sequences among many different species. It is as if MHC genes are horizontally transferred among closely related hosts, which might be feasible given that MHC sequences are horizontally transferred from hosts to pathogens (Imase *et al.*, 2001). However, we are unaware of studies that have considered this idea. These various hypotheses constructed to explain the evolutionary origins of MHC diversity are not mutually exclusive, and they will be difficult to distinguish (Sette *et al.*, 2003).

Some species lack polymorphic MHC loci, and several hypotheses have been proposed to explain why: (1) selective sweeps driven by infectious diseases; (2) genetic bottlenecks and drift; (3) reduced pathogens in solitary species and aquatic mammals (Slade, 1992; Bowen *et al.*, 2004); and (4) limited gene flow among populations. Island populations are likely to have reduced MHC diversity, as found in Australian bush rats (*Rattus fuscipes*) (Seddon and Baverstock, 1999). As more MHC studies are published on different species, comparative approaches will help understand the evolutionary origins and maintenance of MHC diversity. They can be used, for example, to test whether MHC polymorphisms are elevated among species exposed to more infectious diseases (or greater fluctuations) (Nevo and Beiles, 1992), as predicted by PMS, or species showing MHC-dependent mating preferences, as predicted by SS (Penn and

Potts, 1999). Inter- and intraspecific variation in MHC polymorphisms may also be influenced by social structure (e.g. social versus solitary) (Faulkes *et al.*, 1990; Hambuch and Lacey, 2002). Comparative approaches must take into account phylogeny, population size, and historical bottlenecks, which can be a challenge. It would be of practical importance to know whether low levels of MHC diversity increases the risk of extinction, as this question has implications for endangered species and conservation (O'Brien and Evermann, 1988; Hughes and Nei, 1992; Radwan *et al.*, 2010).

Non-classical MHC genes (Box 9.2) are monomorphic in laboratory mouse strains; however, there are surprisingly few studies on these genes in wild mice, as most studies of the MHC region have focused on the immunological functions of classical class I and II molecules (Ohtsuka *et al.*, 2008). As we explain below, non-classical MHC genes appear to evolve from classical MHC genes, and the latter duplicate with a high frequency (Hughes and Nei, 1989). Non-classical MHC genes are often silenced and non-functional, though some perform various functions in the immune system and reproduction (e.g. non-classical MHC genes are expressed on the placenta). Recent studies discovered that non-classical Ib MHC genes in mice regulate the expression of receptors of the vomeronasal organ (VNO), which controls pheromone detection (reviewed in Hedge, 2003; Ishii *et al.*, 2003; Loconto *et al.*, 2003). Studies are needed to examine polymorphism of non-classical MHC loci, and determine how they evolve new functions.

Early studies on the evolution of MHC genes relied mainly on genetic data, coalescence theory, and comparisons of synonymous *versus* non-synonymous substitutions to test for selection over macro-evolutionary timescales (Hughes and Nei, 1988; Takahata and Nei, 1990) and to examine their evolutionary origins. Bioinformatic approaches using the large amounts of genomic data increasingly available will surely continue along these lines. These approaches are important for understanding the history of MHC genes; however, they shed little light on the nature of selection on MHC genes (Apanius *et al.*, 1997; Garrigan and Hedrick, 2003; modelling approach in Ejsmond *et al.*, 2010; Spurgin and Richardson, 2010). Understanding how selection maintains MHC diversity requires studying fitness of animals living in the wild or semi-natural conditions. Before considering how selection maintains MHC polymorphisms, however, we examine a closely related aspect of diversity, the evolution of MHC gene duplications.

The evolution of MHC gene duplications

Until around 1990, most multigene families were thought to be subject to concerted evolution, in which all member genes of a family evolve as a unit in concert. However, phylogenetic analysis of MHC and other immune system genes showed a quite different

evolutionary pattern, and a new model called birth-and-death evolution was proposed. (Nei and Rooney, 2005)

MHC genes are a multiple-gene family and the evolution of gene duplications has also been difficult to explain (Innan and Kondrashov, 2010). The number of MHC loci can vary greatly among different species, and it is unclear why. Although duplicate genes may only rarely evolve new functions, the stochastic silencing of such genes may play a significant role in the passive origin of new species (Lynch and Conery, 2000). Generation of new alleles can be achieved via gene conversion events or via birth-and-death evolution (Nei and Rooney, 2005). Birth-and-death evolution produces new orthologous genes by duplication, the duplicates diverging by accumulating mutations over time. Some of these duplicate genes persist in the genome, and some are deleted or become pseudogenes (Nei and Rooney, 2005). Duplicated MHC genes can be found in a wide range of species (e.g. Miller and Lambert, 2004; Miska *et al.*, 2004; Reusch *et al.*, 2004; Harf and Sommer, 2005; Baker *et al.*, 2006; Bryja *et al.*, 2006; Schwensow *et al.*, 2007) and support the idea that gene duplication is an important process in MHC evolution.

Many species show copy number variation (CNV) for MHC loci, though to our knowledge there has been little work on this area in house mice. A comparison of two different inbred strains of mice suggested that the number of class I genes vary by up to 20% among different haplotypes (Rogers *et al.*, 1985). Interestingly, a recent study found that *M. m. musculus/domesticus* hybrids show increased CNV in the genome (Scavetta and Tautz, 2010).

Understanding the evolution of MHC gene duplication and maintenance of CNV should help shed light on the selective maintenance of MHC polymorphisms – and vice versa. Heterozygote advantage, for example, is suspected to provide positive selection for new gene duplications, as well as promoting polymorphisms, since MHC heterozygosity often enhances disease-resistance and fitness (Penn *et al.*, 2002) (see below). The strength of selection for duplications should be similar to the amount favouring heterozygotes (Otto and Yong, 2002). Natural selection may favour individuals with an intermediate rather than maximum number of MHC molecules for immune function ('optimal MHC heterozygosity' hypothesis) (reviewed in Penn and Potts, 1999; Woelfing *et al.*, 2009). Evidence for optimizing selection on individual allele number is insufficient to explain the evolution of MHC polymorphisms (Hedrick, 2004); however, it shows *stabilizing selection* on the number of MHC loci. Studies are needed to test whether the optimum number of individual allelic diversity varies in space or time due to fluctuating selection (see below). Mating preferences for MHC heterozygotes or individuals with high allelic diversity (Griggio *et al.*, 2011;

Thoß *et al.*, 2011) may also provide positive or fluctuating selection for new gene duplications. Sexual selection may reinforce PMS, favouring new disease-resistant mutations at new duplications and stabilizing selection for an intermediate or optimum number of loci.

To understand the evolution of duplications, it is necessary to determine the various functions of MHC molecules. Although MHC genes are generally assumed to function primarily or exclusively for antigen presentation to T cells, studies with house mice show that MHC genes are also involved in many other – and completely unexpected – functions, including individual odour (Yamazaki *et al.*, 1990; reviewed in Penn and Potts, 1998b), commensal microflora communities (Lanyon *et al.*, 2007), pregnancy rejection (Rülicke *et al.*, 1998), iron metabolism (Cardoso *et al.*, 2002), pheromone detection (reviewed in Hedge, 2003; Ishii *et al.*, 2003; Loconto *et al.*, 2003), and the development and function of cortical neurons in the brain (Zohar *et al.*, 2008). Some, though not all, of these studies have ruled out potential confounding effects due to genes linked to the MHC – further work is needed here. Nevertheless, these findings have important implications for understanding the evolution and the consequences of MHC diversity and gene duplication. Moreover, it has been suggested that *promiscuous functions* of an existing protein (also known as 'cross-reactivity' or 'moon-lighting activity') can provide a selective advantage in changing environments, allow for the evolution of new gene functions, and enhance evolvability (Otto and Yong, 2002; Aharoni *et al.*, 2005). Since MHC genes are functional for more than antigen presentation, it is necessary to consider how these other functions influence selection. Before considering the possible role of sexual selection, however, we first examine selection from pathogens.

Pathogen-mediated selection

> [T]he struggle against disease, and particularly infectious disease, has been a very influential evolutionary agent, and some of its results have been rather unlike those of the struggle against natural forces, hunger, and predators, or with members of the same species . . .. The most that the average species can achieve is to dodge its minute enemies by constantly producing new genotypes. (Haldane, 1949)

There is much evidence that MHC genes influence resistance to pathogens and parasites, especially in laboratory mice (Apanius *et al.*, 1997; Penn, 2002), though surprisingly little evidence that pathogens impose balancing selection in wild populations (also see Goüy de Bellocq *et al.*, Chapter 18 in this volume, for recent studies challenging the hypothesis that *M. m. musculus/M. m. domesticus* hybrids are susceptible to parasites). Several ideas have been proposed for how selection

from pathogens might drive MHC diversity; however, it will not be easy to distinguish between these non-mutually exclusive hypotheses (Spurgin and Richardson, 2010).

Group selection

H-2 polymorphism prevents [extinction of the species] .... It assures the existence in a population of at least some individuals with the right H-2 alleles and the right T-cell repertoire to enable activation of defense reactions to any pathogen. (Klein, 1979)

It was once widely assumed that MHC polymorphisms evolved as a way to protect the species or populations from extinction, but it is increasingly recognized that such group selection is inadequate to explain MHC diversity. Group selection may favour populations and species with greater MHC diversity; however, it cannot overcome the process of individual or genetic selection eroding diversity within groups. If selection within populations promotes MHC diversity, group selection is unnecessary. This argument does not mean that group selection does not play a role, and should be discounted. Group selection might contribute to diversification (e.g. such multi-level, balancing selection as has been used to explain the evolutionary maintenance of T alleles in house mice), but it is not a sufficient explanation. Thus, the main goal is to determine how selection operates on MHC diversity within populations.

Heterozygote advantage

This idea of heterozygote advantage – or overdominance – is still central to MHC research. (Meyer and Thomson, 2001)

It is often suggested that MHC diversity is maintained by heterozygote advantage (Doherty and Zinkernagel, 1975; Hughes and Nei, 1988; Takahata and Nei, 1990). This hypothesis assumes that MHC heterozygotes (*ab*) have higher fitness than either of the parental homozygotes (*aa* or *bb*). To avoid confusion, this synergistic effect is better labelled 'heterozygote superiority' or 'overdominant selection', as explained below. This idea is based on the expectation that MHC heterozygotes present a greater diversity of antigens to the immune system and cover more immunological 'blind spots' than homozygotes (Doherty and Zinkernagel, 1975). Immunological studies, primarily with house mice, however, paint a more complicated picture. MHC heterozygosity may broaden the array of antigens for TCR; however, it may also have negative effects that favour intermediate rather than maximal levels of MHC heterozygosity ('optimal MHC heterozygosity' hypothesis) (reviewed in Penn and Potts, 1999; Woelfing *et al.*,

2009). MHC genes influence the development of the TCR repertoire, and increasing the number of different MHC alleles expressed may eventually have negative effects on many aspects of immunity (TCR diversity, regulatory T cells, immunopathology, etc.). The development of the TCR repertoire is influenced by both MHC and background genes (Vukusic *et al.*, 1995), and it appears to depend mainly on the diversity of antigens presented by MHC molecules (Matsutani *et al.*, 2011). Therefore, the benefits of MHC heterozygosity may decline with increasing levels of background or overall heterozygosity in the genome (Ilmonen *et al.*, 2007; Thoß *et al.*, 2011).

Observational and experimental studies of mice show that MHC heterozygosity enhances host resistance to most pathogens and parasites; however, contrary to what is often assumed, these studies provide few examples of MHC *heterozygote superiority* (Williams *et al.*, 1978; McLeod *et al.*, 1989; reviewed in Penn, 2002). Most experimental studies show that MHC-dependent disease resistance is generally *dominant*, rather than overdominant (i.e. heterozygotes are as resistant as the best parental homozygote, but not more so; see Fig. 9.2). MHC-dependent resistance can also be co-dominant for some pathogens, which provides no HA (Penn, 2002; Wedekind *et al.*, 2005, 2006), and recessive, which can result in a heterozygote *disadvantage* (Penn, 2002; Ilmonen *et al.*, 2007). Dominance is the most common pattern, and it is sufficient to provide heterozygotes an advantage compared to the average parental homozygote (Penn, 2002). Therefore, dominance may explain cases of HA in observational studies with wild, outbred species, indicating a need for allele-specific measures in future studies (Penn, 2002; Lipsitch *et al.*, 2003). Such 'heterozygote advantage through dominance' will not generate the balancing selection required to maintain MHC diversity, although it could provide a selective advantage for MHC-disassortative mating preferences (Penn, 2002). When resistance is dominant, overdominance may emerge during multiple infections, especially with combinations of pathogens showing reciprocal resistance profiles (Apanius *et al.*, 1997; Penn and Potts, 1999). One experiment found support for this idea (McClelland *et al.*, 2003), but more studies are needed to compare the relative *fitness* of MHC genotypes as well as pathogen clearance. For some infectious diseases, MHC heterozygotes can be high immune responders, but are less likely to survive the infection compared to homozygotes (due to immunopathology) (Doherty and Zinkernagel, 1975).

Studies measuring fitness of wild-derived house mice indicate that MHC heterozygosity can increase or reduce fitness depending upon exposure to infectious agents and genetic background. First, a study on congenic strains of mice found that MHC heterozygosity enhanced pathogen clearance and host survival (against multiple strains of *Salmonella*) (Penn *et al.*, 2002). HA was due to resistance being dominant, and not overdominant, as in most previous studies.

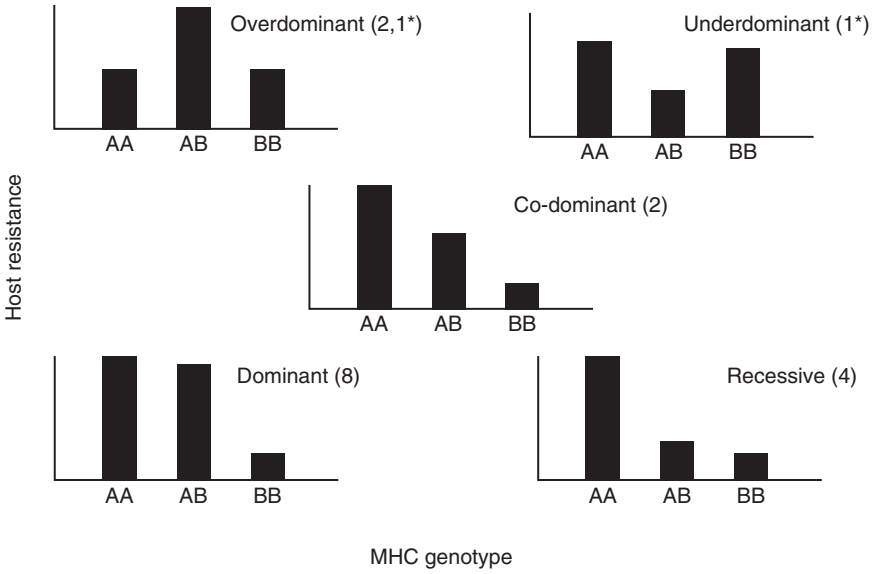

Figure 9.2 Experimental infection studies with laboratory mice show that MHC-dependent resistance to infection is generally (a) dominant (eight studies), rather than (b) overdominant (one or two studies), (c) co-dominant (two studies), (d) recessive (four studies), or (e) under-dominant (either zero or one study) (Penn, 2002). The study usually cited in support of overdominant selection found that MHC heterozygous mice present more antigens to T cells than homozygotes; however, the heterozygous mice died in the experiment, apparently from over-responsiveness (Doherty and Zinkernagel, 1975). Subsequent studies have found that MHC-dependent resistance to infection can be dominant (Penn *et al.*, 2002; McClelland *et al.*, 2003) or co-dominant (Wedekind *et al.*, 2006), and a study with semi-wild mice found that resistance to infection was recessive, which resulted in heterozygote disadvantage (Ilmonen *et al.*, 2007).

Similar results were found when antiviral defences were examined in *bm* mutant mice, which only differ genetically from controls by four amino acid substitutions in the peptide-binding region of class I molecules (Messaoudi *et al.*, 2002). Second, a study on semi-wild mice (wild × MHC-congenic crosses) found no evidence that MHC heterozygosity enhanced resistance to *Salmonella* infection (resistance was recessive); on the contrary, MHC-heterozygous females had reduced numbers of offspring compared with homozygotes (Ilmonen *et al.*, 2007). This result may have been due to the genetically heterozygous background or testing animals with maximal levels of MHC heterozygosity (optimal heterozygosity hypothesis). Third, a recent study on wild mice (F_2 from wild-caught *M. m. musculus*) living in large population enclosures found that MHC

heterozygosity enhanced individual mating and reproductive success (Thoß *et al.*, 2011). This finding was not confounded by rare MHC alleles (there were none) or inbreeding (close inbreeding was controlled through experimental outbreeding of the mice and confirmed with genetic markers). It is crucial to control these factors when studying outbred animals since MHC heterozygosity is often correlated with genome-wide or background heterozygosity. This study found an interaction between MHC and background heterozygosity, such that the benefits of MHC heterozygosity became more variable and diminished with increasing levels of background heterozygosity (perhaps due to changes in the TCR repertoire by increased antigens presented) (Thoß *et al.*, 2011). Therefore, it is important to study how MHC affects host fitness as a whole, not only survival and resistance to one or a few pathogens, but also including reproductive success in wild, outbred animals living in natural or semi-natural conditions (Box 9.3).

Thus, overdominance rarely occurs, and while dominance can result in HA, this mechanism is insufficient to generate balancing selection by itself (Penn, 2002). Moreover, the models that support overdominant selection require that all heterozygotes and homozygotes have equal fitness (Takahata and Nei, 1990). When this unrealistic assumption is not met, the chances of maintaining polymorphism are reduced and only a few alleles can be maintained (Lewontin *et al.*, 1978; Hedrick and Kim, 2000). Intermediate rather than maximal heterozygosity (or individual allele number) increases resistance to multiple parasites (Wegner *et al.*, 2003), but this form of selection is insufficient to maintain MHC polymorphisms (Hedrick, 2004). On the other hand, HA through dominance or overdominance can still play an important role in contributing to the evolution of MHC polymorphisms, especially through fluctuating selection (see below).

Rare allele advantage (negative frequency-dependent selection)

> I wish to suggest that the selection of rare biochemical genotypes has been an important agent not only in keeping species variable, but also in speciation. (Haldane, 1949)

A rather intriguing explanation for how pathogens might drive MHC diversity is through a process of host–pathogen coevolution that results in a 'rare allele advantage' or 'negative frequency-dependent selection' (FDS) (Bodmer, 1972; Clarke, 1979; Takahata and Nei, 1990; Borghans *et al.*, 2004). Since viruses and other pathogens often have much shorter generation times than their hosts, they can potentially evolve adaptations around their hosts' immune defences. If pathogens tend to adapt most rapidly to common MHC genotypes, which seems a reasonable assumption, this process will tend to provide a selective advantage for rare genotypes. Consequently, hosts with rare, resistant MHC

Box 9.3 The importance of studying wild house mice in natural or semi-natural conditions

Although studies on domesticated house mice in the laboratory have been extremely useful for understanding how MHC genes influence immune resistance to pathogens, odour, and other phenotypes, it is unclear whether and how these findings can be extrapolated to wild house mice. First, domesticated, laboratory strains of house mice kept in conventional conditions suffer from many health problems (Martin *et al.*, 2010), and there are significant differences in their behaviour and physiology compared to their wild, outbred counterparts (Crawley *et al.*, 1997; Miller *et al.*, 2002). Second, MHC genes can have different effects depending on the genetic background (G × G interactions), and whenever inconsistent results are found among strains, it is impossible to generalize without testing outbred animals. Third, MHC effects might only become apparent, for example, when studied on a homozygous background, when they are the only loci in the genome that differ among individuals. Additionally, determining how natural selection maintains MHC diversity in wild populations requires studying the survival and reproductive success (Darwinian fitness) of animals living in natural or semi-natural ecological conditions. For these reasons, researchers have studied MHC effects on fitness of mice living in semi-natural conditions, not only with congenic strains (Penn *et al.*, 2002), but also with 'semi-wild' (from domesticated × wild crosses) (Potts *et al.*, 1991; Penn and Potts, 1999; Ilmonen *et al.*, 2007) and 'wild-derived' house mice (recently trapped in the wild and reared in the laboratory) (Sherborne *et al.*, 2007; Thoß *et al.*, 2011). We know of no studies that have addressed how MHC genes influence fitness in wild, free-ranging house mice, whose survival and reproduction is not influenced by researchers, and such studies might prove to be most informative. We would emphasize, however, that since studies on free-ranging animals also involve human observers, trapping, marking, sampling, they are 'semi-natural' as well, and differ only in the relative degree or type of human interference. Perhaps the most compelling reason for conducting studies in natural or semi-natural conditions is that fitness effects sometimes only become apparent in competitive settings outside of the laboratory, e.g. inbreeding depression (Meagher *et al.*, 2000; Ilmonen *et al.*, 2008); selection against segregation distorters (t alleles; Carroll *et al.*, 2004); and effects of MHC heterozygosity on reproductive success (Ilmonen *et al.*, 2007; Thoß *et al.*, 2011).

alleles evolve to a higher frequency over time, which then would provide a selective advantage for pathogens to adapt to the new common MHC allele. This process of reciprocal adaptation can theoretically result in continual co-evolutionary cycles, and maintain genetic polymorphisms in both hosts and pathogens (models reviewed in Apanius *et al.*, 1997; Hedrick and Kim, 2000; Tellier and Brown, 2007). Host–pathogen coevolution can easily account for polymorphisms of more than 50 alleles per MHC locus (Borghans *et al.*, 2004).

There is evidence for FDS on MHC alleles in human HIV studies (Moore *et al.*, 2002; Trachtenberg *et al.*, 2003); however, there is no direct evidence in house mice or any other species to our knowledge. Many studies, mainly of laboratory mice, have discovered a variety of mechanisms through which pathogens can adapt to host MHC genotypes, such as by escaping antigen presentation (Potts and Slev, 1995). Some pathogen adaptations that allow evasion of MHC-dependent recognition, such as downregulating of MHC expression, may not cause selection on the antigen-binding site, though they might still play a role in driving MHC diversity. However, to our knowledge, there is no evidence for genotype-specific resistance and infectivity between host MHC and pathogen alleles, as required for FDS, or models showing that reciprocal coevolution can occur even though host–parasite interactions are generally 'diffuse' (Thompson, 1989). FDS will be difficult to test and to distinguish from other models, as they share several predications (Apanius *et al.*, 1997; Hedrick, 2002; Spurgin and Richardson, 2010).

Fluctuating selection over time or space

> We know virtually nothing about the role of spatial and temporal heterogeneity of environment in evolution and it is here that the most fruitful and most difficult area of research lies. (Lewontin, 1964, cited in Hedrick and Kim, 2000)

The most realistic hypothesis for how pathogens drive MHC diversity is probably through temporal or spatial fluctuations in selection (FS) (Hill *et al.*, 1991; Hedrick, 2002; for general overview of models see Gillespie, 1991; Thompson, 2005). If parasites vary geographically – either in species presence, density, or genetically – over their hosts' range, then in such a mosaic landscape different MHC alleles will be selectively favoured in different populations. MHC diversity can become reduced within populations as a result of selective sweeps, but MHC diversity among host populations should be maintained. Similarly, if parasites vary over time, then fluctuating temporal selection can also maintain MHC diversity (even without FDS or other forms of host–pathogen coevolution). FS in time or space provides broader conditions for maintaining polymorphisms than in constant environments. The conditions favouring diversity are generally more robust for spatial than temporal variation (Hedrick, 2006), though both are likely to occur and may interact (pathogens in some locations may change more over time than others).

Hedrick (2002) examined a model that assumes different MHC alleles confer resistance to different pathogens (specific resistance alleles), resistance to infection is dominant, and that pathogens fluctuate over time in terms of their presence or

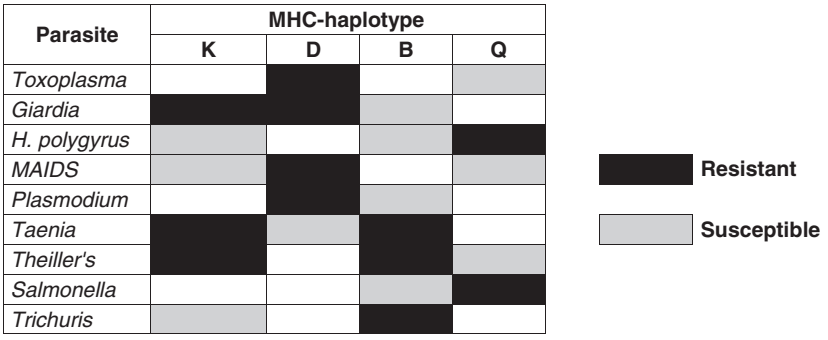

Figure 9.3 Studies on house mice show that MHC haplotypes that confer resistance to one parasite often increase susceptibility to other parasites, which supports the idea that parasites impose balancing selection on MHC genes (see references in Apanius *et al.*, 1997, Table 3).

absence. This model indicates that fluctuating temporal selection can maintain high levels of MHC polymorphisms, and requires no FDS or 'intrinsic heterozygote advantage' (overdominance). It is unclear whether fluctuations in pathogens are sufficient to explain MHC diversity in house mice, though studies on laboratory house mice provide support for the assumptions in this model. First, a survey of experimental studies on MHC-dependent resistance to infectious diseases found that MHC haplotypes that enhance resistance to one pathogen in one study often increase susceptibility to other pathogens in other studies (allele-specific resistance/susceptibility tradeoffs) (Fig. 9.3) (Penn and Potts, 1999; Penn *et al.*, 2002). Such tradeoffs were confirmed with experimental infections (McClelland *et al.*, 2003). Second, experimental studies with mice show that MHC-dependent disease resistance is generally dominant (Penn, 2002; Penn *et al.*, 2002; McClelland *et al.*, 2003) (Fig. 9.2). More work is needed, however, to examine whether parasites show sufficient spatial or temporal fluctuations to drive MHC diversity (see review of the evidence in Piertney and Oliver, 2006).

The finding that MHC alleles confer resistance tradeoffs with different pathogens (Penn and Potts, 1999; Penn, 2002; Penn *et al.*, 2002; McClelland *et al.*, 2003) provides evidence for balancing selection on MHC diversity. Such balancing selection is not sufficient, however, to maintain high levels of MHC diversity under realistic conditions (unequal fitness among homozygotes and among heterozygotes). Some other factor is necessary, such as fluctuations in the presence/absence of pathogens (though environmental fluctuations do not solve the problem by generating $G \times E$ interactions, contrary to what is often suggested (see Hedrick, 2006)).

The various hypotheses for PMS are not mutually exclusive and there are reasons to expect that they may interact and reinforce each other. For example, spatial and temporal fluctuations can stabilize host–parasite coevolutionary dynamics and help to maintain genetic diversity in hosts and pathogens (Sasaki *et al.*, 2002). It is still unclear whether PMS is adequate to explain high levels of MHC diversity, especially since other immune recognition loci do not appear to be highly polymorphic. Increasingly, researchers are considering the possibility that sexual selection may also play a role in driving MHC diversity, and in the next section we review the evidence in house mice.

Sexual selection

> These mating preferences could in natural populations serve the purpose of increasing the representation of particular H-2 haplotypes or of maintaining heterozygosity of genes in the region of H-2. (Yamazaki *et al.*, 1976)

Studies on house mice have found that MHC genes influence odour (reviewed in Penn and Potts, 1998b; Penn, 2002; Beauchamp and Yamazaki, 2003; Yamazaki and Beauchamp, 2007; Kwak *et al.*, 2010) and disassortative mating preferences (reviewed in Penn and Potts, 1999; Penn, 2002; Yamazaki and Beauchamp, 2007; Roberts, 2009). MHC disassortative preferences can potentially drive MHC polymorphisms, though there is still no direct evidence from populations of wild house mice. It has been suggested that MHC-dependent mating preferences in mice 'could not be much more than a quirk of nature without general significance' (Klein *et al.*, 1993). However, several recent studies have found evidence for MHC-dependent mating preferences in other taxa, including fish, reptiles, and even birds (Zelano and Edwards, 2002; Bernatchez and Landry, 2003; Milinski, 2006; Havlicek and Roberts, 2009). The most interesting implication of these studies is that they provide a potential example of how mating preferences can generate selection and evolution of genes that control immune recognition of pathogens and parasites.

Mating preferences

Yamazaki and his colleagues (1976, 1978) performed the pioneering studies that first found evidence that MHC genes influence odour and mating preferences. They studied MHC-congenic strains and found that males preferred to mate with females from MHC-dissimilar strains. However, since only some strains – and only males – showed such mating preferences, it remained unclear whether this behaviour even occurs in wild mice. Brown and his

colleagues found that female laboratory mice displayed MHC-disassortative odour and mating preferences (Egid and Brown, 1989), but found no evidence with males (Eklund *et al.*, 1991). Potts and his colleagues (1991) studied populations of (semi-wild) mice living in semi-natural conditions and found that half the litters contained offspring sired by males other than the dominant, territorial male. Among these litters, MHC loci were more heterozygous than expected if females had mated only with the territorial male. This finding suggested that females sought extra-pair copulations with more-MHC-disparate males.

Yamazaki *et al.* (1988) found that males' MHC-mediated mating preferences could be reversed by rearing them with MHC-dissimilar families (cross-fostering). This effect was also found in female mate preferences in laboratory strains (Eklund, 1997a) and to a much lesser extent in wild female mice (Eklund, 1997b). This finding indicated that mice learn the MHC-identity of their family ('familial imprinting') and avoid mating with partners that have familial MHC genes. Penn and Potts (1998c) tested this idea in (semi-wild) female mice. Behavioural observations and genotyping offspring indicated that females avoid mating with MHC-similar males, unless they were cross-fostered at birth; then they avoided males similar to their family. These findings provide direct evidence for MHC-dependent mating preferences in females living in semi-natural conditions, and support the familial imprinting hypothesis.

Two recent studies raise questions about whether MHC genes influence mating biases in wild populations of house mice. Roberts and Gosling (2003) found that female mice are attracted to the scent marks of MHC-dissimilar males, but only when scent-marking rate was similar between stimulus males (and females' preference was better explained by males' marking rate than by MHC sharing). These findings provide evidence that females are attracted to the scent of MHC-dissimilar males, but also raise the possibility that such odour biases may not affect mating patterns in natural conditions where males vary in their scent marking. Another study compared the relative importance of MUP (major urinary proteins) versus MHC genes for inbreeding avoidance in wild-derived mice in semi-natural enclosures (Sherborne *et al.*, 2007). The mice could choose between full versus half-siblings (no option to avoid inbreeding), and genetic analyses of offspring were used to assess mating preferences. The authors found a deficit in successful matings (based on number of offspring) between mice sharing MUPs, but not MHC haplotypes, and concluded that inbreeding avoidance is based on MUP rather than MHC sharing. This conclusion is premature for several reasons, however. First, the results indicate that there was no inbreeding avoidance in this study (no bias to mate with half versus full-sibs), which is necessary to conclude that MUPs but not MHC genes influence inbreeding avoidance. Second, the mice may have avoided mating with familial

MHC genotypes (Yamazaki *et al.*, 1988; Penn and Potts, 1998c), but familial imprinting was not considered. Third, before being released into the enclosures, the mice were reared since birth together in cages with individuals carrying all the MHC haplotypes later encountered in the enclosures. Consequently, the mice were forced to choose their mates among individuals that potentially had already been classified as kin through familial imprinting (which might explain why there was no inbreeding avoidance) (Ruff *et al.*, 2011). Although MUPs play an important role in chemical communication (Hurst, 2009), there are only a few attempts to assess their role compared to MHC (Brennan, 2001; Sherborne *et al.*, 2007; Thom *et al.*, 2008; see also Stopka *et al.*, Chapter 8).

Cryptic female choice

MHC may also play a role in post-copulatory (cryptic) female choice in house mice and other species (Wedekind, 1994). There is mixed evidence for non-random fertilization success of gametes of different MHC haplotypes, though this effect appears to depend on a female's health or infection status (Wedekind *et al.*, 1996; Rülicke *et al.*, 1998). The mechanism may be controlled by egg–sperm interactions or perhaps modulated by olfactory receptor (OR) genes (Ziegler *et al.*, 2002) or other MHC-linked genes. MHC genes do not appear to be expressed at this stage, and yet in humans selection maintains a linkage disequilibrium between OR and MHC loci (Santos *et al.*, 2010). The sperm receptor selection hypothesis (reviewed in Ziegler *et al.*, 2005, 2010) proposes that during spermatogenesis the expression of chemoreceptors (such as olfactory receptors or VNO receptors) is modulated through elimination of self-reactive receptors, resulting in the generation of sperm that are sensitive only to non-self-MHC antigens. Ligand binding affects sperm motility and thereby fertilization potential. While the egg does not express MHC molecules itself, the surrounding granulose cells do, thereby shedding antigens into the follicular fluid (Dohr *et al.*, 1987), which could interact with approaching sperm.

Not all studies find MHC-dependent mating (pre- or post-copulatory) preferences, and it would be interesting to know if there are differences among geographical populations maintained by fluctuating selection. Also, individual preferences can change as mating preferences are often conditional, and expressed only under certain circumstances. For example, females may pay attention to the MHC of potential mates when seeking extra-pair mates, and especially when their own mates are genetically similar to themselves. One of the main problems with the sexual selection hypothesis, however, is that it is difficult to understand how MHC genes influence odour (reviewed in Penn and Potts, 1998b; Penn, 2002; Beauchamp and Yamazaki, 2003; Yamazaki and Beauchamp,

2007; Kwak *et al.*, 2010). MHC genes clearly influence odour, but how remains a mystery (see Stopka *et al.*, Chapter 8). In addition to determining the mechanisms underlying MHC-mediated mate choice, there is increasing interest in determining the underlying functions.

Evolutionary functions of MHC-dependent mating preferences

Two main types of hypotheses have been suggested to explain the potential functions of MHC-dependent mating preferences. MHC-disassortative mating preferences may be selectively favoured to: (1) enhance offspring resistance to pathogens through several possible mechanisms; (2) facilitate inbreeding avoidance; or both (reviewed in Penn and Potts, 1999; Penn, 2002). These hypotheses are not mutually exclusive, as inbreeding avoidance may also enhance offspring resistance to infectious diseases (Keller and Waller, 2002; Ilmonen *et al.*, 2009).

There are two main ways that MHC-disassortative mating preferences have been suggested to enhance offspring resistance to pathogens. First, MHC-disassortative mating preferences might function to produce disease-resistant, MHC heterozygous offspring (Penn and Potts, 1999; Penn *et al.*, 2002). As addressed above, the evidence for this idea shows that MHC heterozygosity often (but not always) enhances disease-resistance and fitness, and more studies are needed with wild or wild-derived house mice (Thoß *et al.*, 2011; see also Goüy de Bellocq *et al.*, Chapter 18). Similarly, MHC-dependent mating preferences might help optimize rather than maximize offspring MHC heterozygosity (Penn and Potts, 1999; Penn *et al.*, 2002) or individual allelic diversity (Reusch *et al.*, 2001; Aeschlimann *et al.*, 2003). Second, MHC-disassortative mating preferences may provide a 'moving target' against pathogens adapted to the parental genotypes (Penn and Potts, 1999). This 'moving target' hypothesis is supported by a computer simulation (Howard and Lively, 2002), and by an experiment with congenic house mice, which found that MHC heterozygotes can more effectively resolve co-infections of pathogens that are resistant to the immune defences of parental MHC genotypes (McClelland *et al.*, 2003).

MHC-disassortative mating preferences might function to help avoid kin matings (inbreeding avoidance) (Apanius *et al.*, 1997; Penn, 2002). Some studies find that house mice avoid kin matings (Winn and Vestal, 1986; Krackow and Matuschak, 1991), and there can be surprisingly strong selection against inbreeding (Barnard and Fitzsimons, 1989; Meagher *et al.*, 2000; Ilmonen *et al.*, 2008). The benefits from inbreeding avoidance appear to be greater than those from enhancing offspring MHC-heterozygosity (Potts *et al.*, 1988). The inbreeding avoidance hypothesis suggests that MHC genes play a role in kin recognition, though only a few studies have tested this idea with mice. Female mice

sometimes nest communally, and a study with semi-wild mice found that females nest with MHC-similar females when siblings are unavailable (Manning *et al.*, 1992). Another study, however, found no evidence for MHC-similar odour preferences among congenic strains of female mice (Ehman and Scott, 2001). Yamazaki *et al.* (2000) studied parent–offspring recognition with MHC congenic mice, and found that females are more likely to retrieve pups when they are MHC-similar, and pups are attracted to the odours of mothers that are MHC-similar. This study provides direct evidence that MHC genes play a role in kin recognition, but there have been no such studies on wild mice to our knowledge.

There are other ways that SS can potentially play a role in promoting MHC diversity, besides disassortative mating preferences. For example, females often prefer to mate with disease-resistant males (Hamilton and Zuk, 1982), which will likely carry disease-resistant MHC alleles ('good genes' sexual selection) (von Schantz *et al.*, 1989; Penn, 2002; Eizaguirre *et al.*, 2009). Such directional mating preference for health or condition should enhance offspring fitness and reinforce PMS (FDS or FS) on MHC diversity. This idea has not been tested in house mice, but it is consistent with evidence that females prefer the scent of healthy versus infected males (Kavaliers and Colwell, 1995; Zala *et al.*, 2004) (reviewed in Penn and Potts, 1998a) and genetically resistant versus susceptible males (Zala *et al.*, 2008). Furthermore, females may be attracted to disease-resistant, MHC-heterozygous males, and there is some evidence for this idea in house mice (Thoß *et al.*, 2011). It is unclear whether preferences for MHC heterozygotes confer genetic benefits, though such preferences would reinforce PMS favouring heterozygotes.

Thus, the hypothesis that SS plays a role in shaping MHC diversity has been gaining support, but the evidence is still mixed in house mice and other species. The SS hypothesis has been controversial and sometimes treated with much scepticism, though sometimes due to misunderstandings (Box 9.4).

Outlook: functional evolutionary genomics and integrating PMS and SS

> Because of the richness of data for this system and the apparent importance of a number of evolutionary factors affecting variation at [MHC loci], we feel that [the MHC] has become an exemplary system for understanding evolutionary genetics. (Hedrick *et al.*, 1987)

There is growing interest in trying to understand the evolutionary origins and selective maintenance of MHC genetic diversity (polymorphisms and duplications). It is unclear why this intriguing problem has not attracted more attention

Box 9.4 Why the sexual selection hypothesis is often treated with scepticism

The idea that SS might help explain the evolution of MHC polymorphisms has been controversial, but some of the scepticism is based on misunderstandings. For example, sometimes SS studies are misinterpreted as claims that mate choice is controlled solely by MHC genes. This misunderstanding may help explain why human studies have been controversial (reviewed in Roberts, 2009). Also, as we previously pointed out, the SS hypothesis is sometimes mistakenly assumed to be a mutually exclusive alternative to PMS, although these ideas are completely compatible (Penn and Potts, 1999).

Additionally, the SS hypothesis is often perceived as far-fetched. It is not always intuitive that mating preferences can function to produce disease-resistance offspring or provide an important source of selection, driving evolutionary genetic changes. Nevertheless, theoretical models support the idea that pathogens can drive the evolution of disassortative mating and other forms of non-random mating preferences (Howard and Lively, 2003; Nuismer *et al.*, 2008). In fact, the leading explanation for the evolutionary function of sexual reproduction itself is to allow hosts to 'keep up' in a never-ending co-evolutionary race with rapidly evolving pathogens and parasites (Red Queen hypothesis) (Hamilton, 2001). There is increasing evidence that mate choice in many species, including house mice, can function to provide genetic benefits for offspring (reviewed in Hettyey *et al.*, 2010), including resistance to infectious agents (Hamilton and Zuk, 1982). Thus, from an evolutionary perspective, the SS hypothesis is not as extraordinary as some assume.

Regardless of evolutionary theory and empirical evidence, the SS hypothesis will continue to be viewed as far-fetched by some researchers until the underlying mechanisms are better resolved. There is much evidence that MHC genes influence odour, and though there are several potential mechanisms, it remains a major challenge to determine how this occurs (reviewed in Penn and Potts, 1998b; Penn, 2002; Beauchamp and Yamazaki, 2003; Yamazaki and Beauchamp, 2007; Kwak *et al.*, 2010; see also Stopka *et al.*, Chapter 8). It is feasible that genes in the MHC region *other than class I or II loci* have a more important role in influencing odour and mating preferences, but there has been little work on this alternative. Nonetheless, we cannot rule out mating preferences just because the underlying chemosensory mechanisms are unresolved, no matter how far-fetched this hypothesis might seem. The general emphasis on the immunological functions of MHC genes can blind us to other possibilities, and it has led some to promote group selection and overdominant selection explanations, despite the fact that theoretical (Lewontin *et al.*, 1978; Hedrick and Kim, 2000) and empirical evidence (Apanius *et al.*, 1997; Penn, 2002) have long failed to support these ideas.

in the past – especially given its important consequences for human health – e.g. tissue rejection and resistance to infectious and autoimmune diseases. Biomedical research places a greater emphasis on mechanistic than evolutionary questions, and consequently, the vast majority of studies on MHC genes have been

conducted on immunological mechanisms with domesticated strains of laboratory mice (Box 9.1). This approach has proven useful for unravelling immunological mechanisms controlling resistance to infectious diseases; however, as we previously stressed, determining how natural selection maintains MHC diversity requires studying Darwinian fitness of animals living in natural or semi-natural ecological conditions (functional ecological genomics) (Box 9.3). Testing PMS in the field is a major challenge, especially since it requires tracking changes in host pathogens – and their fitness effects on hosts – over time and space. Future studies on MHC polymorphisms in wild populations would benefit by considering hypotheses about sexual selection, as well as pathogen-mediated selection. One of the most important reasons is that MHC heterozygosity can have significant effects on mating and reproductive success (Darwinian fitness) *even without any discernable effects on viability or survival* in the laboratory (Thoß *et al.*, 2011).

The development of high-throughput DNA-sequencing technologies is exciting and it has prompted an increasing number of studies on MHC polymorphisms in non-model organisms (see Wegner, 2009; Babik, 2010 and references therein). The availability of genomic technologies and bioinformatic analyses will undoubtedly provide new insights into MHC diversity, but there are many caveats and limitations. As Hedrick (2006: 81) points out:

> There appear to be many genomic regions with a history of adaptive selection, but only a small proportion of these indicate a genetic signal consistent with balancing selection. However, the smaller region thought to exhibit a balancing selection signal, compared with a selective sweep, may make detection beyond the resolution of most genomic scans. Further, alleles identified under directional selection may be part of balancing selection at a gene, and further examination of specific alleles may be necessary to clarify the nature of selection.

Perhaps the greatest challenge for studies on the evolution of MHC diversity is obtaining sufficiently *large sample sizes* to detect small selection coefficients. It is ironic that the large diversity of alleles that makes MHC genes so interesting also makes these genes difficult to study due to the large sample sizes necessary to compare fitness of different genotypes. This is problematic because even minimal selection coefficients may nonetheless drive evolutionary changes in MHC polymorphism (Apanius *et al.*, 1997; Meyer and Thomson, 2001).

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